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Cultivar and Environmental Effects on Freezing Tolerance of Narrow-Leaf Plantain

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ABSTRACT

Improved cultivars of narrow leaf plantain (*Plantago lanceolata* L.) have received increasing attention as possible pasture species for the northeastern USA because of their productivity during drought and high nutritive value. However, the cultivars currently available do not have sufficient freezing tolerance to survive northeastern U.S. winters. This experiment examined the relationship between shoot growth rate and root:shoot partitioning during cold-hardening and the freezing tolerance of 'Lancelot' and 'Tonic' plantain compared with 'PG700', an experimental line collected from the eastern USA. I hypothesized that PG700 would exhibit reduced shoot structural growth and increased carbohydrate storage in roots during cold-hardening, resulting in increased freezing tolerance and survival. In growth chamber experiments, seedlings were cold hardened for 21 d and then frozen at −12°C for 3 h. Survival was evaluated after a 21-d recovery period. The experiment was conducted twice. Survival was greatest for PG700 (58%), followed by Lancelot (33%) and Tonic (18%) ($P < 0.01$). When combined across cultivars, survival was 59% in Trial 1 compared with 11% in Trial 2 ($P < 0.01$). None of the measured parameters including overall root and shoot growth or relative partitioning between roots and shoots were related to cultivar differences in freezing tolerance. Reduced survival in Trial 2 was accompanied by high nitrogen uptake and vigorous shoot relative to root growth during the cold-hardening period. Thus, reduced shoot growth was accompanied by increased freezing tolerance when differences in survival were induced by environmental effects but was not related to genetic differences in survival.

Improved cultivars of narrow-leaf plantain have received increasing attention as possible forage and pasture species for the northeastern USA because of their reportedly high productivity during periods of drought and high nutritive value (Stewart, 1996; Rumball et al., 1997). However, the improved cultivars, Lancelot and Tonic, that are currently available were developed in New Zealand from germplasm originating in northern Portugal and the North Island of New Zealand, respectively (Stewart, 1996), and do not appear to have sufficient freezing tolerance to survive northeastern U.S. winters (Sanderson et al., 2001; Skinner and Gustine, 2002). Sanderson et al. (2001) found that plantain died out completely within 2 yr of planting in field trials. Skinner and Gustine (2002) concluded that Lancelot had slightly greater survival than Tonic, but neither cultivar had sufficient freezing tolerance to be recommended for use in the northeastern USA. They further suggested that improved cultivars needed to be developed from populations that have evolved under more severe winter conditions before plantain could become a viable forage for less temperate regions. Naturalized populations of narrow-leaf plantain are found throughout the northeastern USA and could provide parent material for cultivars with improved freezing tolerance.

Plantain grows well under cool temperatures (Chatterton et al., 1990) and is valued in New Zealand for its ability to remain green and leafy during winter (Stewart, 1996). However, in species such as alfalfa (*Medicago sativa* L.), fall dormancy is often closely associated with winter survival (Schwab et al., 1996; Cunningham et al., 2001), such that survival is reduced in cultivars that exhibit vigorous growth during the fall. However, others have shown that fall growth can be dissociated from winter survival (Haagenson et al., 2003b). It remains possible, however, that the poor winter survival of plantain is directly related to its continued growth under environmental conditions that induce fall dormancy in more freezing tolerant species. Indeed, Lancelot, which had the greatest winter survival in a previous study (Skinner and Gustine, 2002), is considered to be a winter dormant cultivar, while Tonic is considered to be more winter active under New Zealand conditions (Stewart, 1996).

Imposition of summer drought in the field increased plantain winter survival to 41% compared with less than 10% survival under normal and excessive soil moisture treatments (Skinner and Gustine, 2002). They also found that plantain growth rates in the normal and wet treatments were greater in September than they were during July. However, growth rates were similar in September and July in the dry treatment. It is possible that the improved winter survival of drought-stressed plantain was directly related to its reduced fall growth compared with well-watered plants.

The purpose of this experiment was to examine the effect that growth rate and root:shoot partitioning during the cold-hardening process has on the freezing tolerance of Lancelot and Tonic plantain compared with PG700, an experimental line developed from collections made in several states in the eastern USA. The working hypothesis was that PG700 would exhibit reduced shoot structural growth and increased partitioning of nonstructural carbohydrates and nitrogen compounds to roots during cold-hardening, resulting in increased freezing tolerance and survival.

MATERIALS AND METHODS

The experimental line, PG700, was developed by Dr. A.V. Stewart at Pyne Gould Guiness, Ltd. in New Zealand from collections of erect plants made in Kansas, North Carolina,

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Abbreviations: TNC, total nonstructural carbohydrates.

Virginia, Maryland, and Tennessee. Seeds of Tonic, Lancelot, and PG700 plantain were germinated at room temperature in Petri dishes, transplanted at 10 seedlings per pot to 15-cm diameter clay pots filled with a vermiculite and sphagnum peat moss potting soil mixture (The Scotts Company, Maryville, OH)[1], and transferred to Conviron PGR15 plant growth chambers (Controlled Environments Ltd, Winnipeg, MB, Canada). Pots were randomly arranged within each chamber. Three growth chambers were used in the study with each chamber constituting a replication and the experiment was conducted twice.

Chamber conditions were initially set at a 14-h photoperiod with a photosynthetically active radiation level of 976 μmol m^{-2} s^{-1} at the beginning of Trial 1 and 906 μmol m^{-2} s^{-1} at the beginning of Trial 2, provided by a combination of high pressure sodium, metal halide, and incandescent lights. Chamber temperature regimes were designed to mimic natural diurnal fluctuations, slowly ramping up from a minimum of 15°C at 600 h to a maximum of 20°C at 1400 h then back to 15°C by 0600 h the next morning. Air temperature was monitored near the top of the plant canopy. Relative humidity also ramped from a high of 75% at 0600 h to a low of 50% at 1400 h then back to 75%. Pots were watered to saturation every 1 to 2 d as needed to prevent water stress. Nutrients were provided as half-strength Hoagland's solution during one of the water applications each week.

Seedlings were grown under these initial conditions for 3 wk, then water was withheld from half the pots for 4 to 5 d. Previous studies found that a 4- to 5-d drought cycle would reduce leaf transpiration rates by about 50% compared with well-watered controls (Skinner and Gustine, 2002). Pots were then watered to field capacity and the drought cycle was repeated two more times. At the end of the third cycle, all pots were watered to saturation and the cold hardening process was started. Chamber temperatures were gradually reduced over a 2-d period to a maximum/minimum of 4/2°C, photoperiod was reduced to 12 h, photosynthetically active radiation was reduced 50% by turning off half the lights, and the humidity control was turned off. With the controls off, humidity ranged from 75 to 85%. This initial hardening period lasted for 2 wk; then air temperature was reduced for an additional week to a maximum/minimum of 2/−1°C, with a 10-h photoperiod. Chamber temperatures were then reduced to −3°C and ice nucleation in the plants was promoted by spraying the plants with cold water. After 24 h at −3°C, the temperature was reduced at a rate of 1°C/h to −12°C. Chambers were kept at −12°C for 3 h, and then the temperature was increased to 4°C at the rate of 2°C/h. After 8 h at 4°C, chamber conditions were returned over a 2-d period to their original, prehardening settings for a 3-wk recovery period. At the end of 3 wk, each individual plant was rated for survival.

At the beginning of the drought cycles, the beginning of cold hardening and the end of cold hardening one pot per accession was removed from each chamber, shoots were clipped at the soil surface, roots were washed free of soil, and all plant material was frozen in liquid nitrogen. Frozen plant materials were freeze-dried and weighed for biomass determinations. Samples were then ground and analyzed for total N content by combusting approximately 7 mg plant material in an EA1100 elemental analyzer (CE Elantech, Lakewood, NJ). Ground samples were also sent to the laboratory of Jeff Volenec at Purdue University for sugar and starch analysis. Sugars were extracted from 30 mg of freeze-dried root tissue with 1 mL of 800 mL L^{-1} ethanol in 1.5-mL microfuge tubes. Tubes were shaken for 10 min at 25°C, microfuged at 14c000 × *g* for 5 min at 4°C, and the supernatant retained. The ethanol extraction was repeated twice and the combined supernatants diluted to a final volume of 10 mL with 800 mL L^{-1} ethanol. Sugar concentrations in the ethanol extracts were determined with anthrone (Van Handel, 1968) using glucose as a standard. The ethanol-extracted residue was oven-dried at 55°C. Water (500 μL) was added to each tube, and the tubes were heated in a boiling water bath for 10 min to gelatinize starch. The pH of the solution was adjusted to 5.1 by adding 400 μL 0.2 *M* Na acetate buffer. Starch was digested by adding 0.2 U of amyloglucosidase (Sigma Chemical Co., St. Louis MO; product A3514 from *Aspergillus niger*) and 40 U of a-amylase (Sigma Chemical Co., St. Louis, MO; product A0273 from *Aspergillus oryzae*) in 100 μL of 0.2 *M* Na acetate buffer (pH 5.1). Tubes were incubated a 55°C for 24 h with occasional shaking. Tubes were centrifuged as before and glucose in the supernatant was determined using glucose oxidase (Glucose Trinder, Sigma Chemical Co., St. Louis, MO; Product 315–100). Starch concentration was estimated as 0.9 × glucose concentration (Cunningham et al., 2001). Total nonstructural carbohydrates (TNC) were calculated as the sum of starch and soluble sugars, and TNC-free dry matter determined by subtracting TNC content from total dry matter.

The experiment was conducted as a 2 × 2 × 3 × 3 factorial (moisture treatment × trial × cultivar × harvest) in a randomized complete block design with the three growth chambers as replications. Root and shoot relative growth rates were calculated following the drought and cold-hardening treatments and analyzed separately for each treatment. Data were analyzed using the SAS procedure Proc GLM (SAS Institute, 1999) and treatment effects considered significant when $P < 0.05$. Mean separation was by protected LSD.

RESULTS

Survival

With the exception of light intensity, chamber conditions were identical for both repetitions of the experiment, yet survival was significantly greater in the first trial compared with the second (Fig. 1). When combined across cultivars, survival was 59% in Trial 1 compared with 11% in Trial 2 ($P < 0.01$). Combined across trials, survival was greatest for the experimental line, PG700 (58%), followed by Lancelot (33%) and Tonic (18%). All differences were significant at $P < 0.01$. There was no significant trial × cultivar interaction. Water stress had less influence than expected on survival although there was a significant trial × cultivar × water interaction ($P = 0.03$). In Trial 1, Tonic survival was 53% in the dry vs. 20% in the wet treatment, whereas, Lancelot survival was 80% in the wet vs. 47% in the dry treatment. Drought had no effect on PG700 survival in either trial or on the survival of Tonic and Lancelot in Trial 2.

Growth

Although drought had minimal effect on survival, both shoot and root TNC content and TNC-free biomass were reduced by the drought treatment when averaged across trials and cultivars, indicating that the plants were water stressed. Root TNC-free dry weight was reduced 31% by the drought treatment compared with a 12%

[1] Mention of a specific brand name is for identification purposes only and does not constitute endorsement by the USDA at the exclusion of other suitable products.

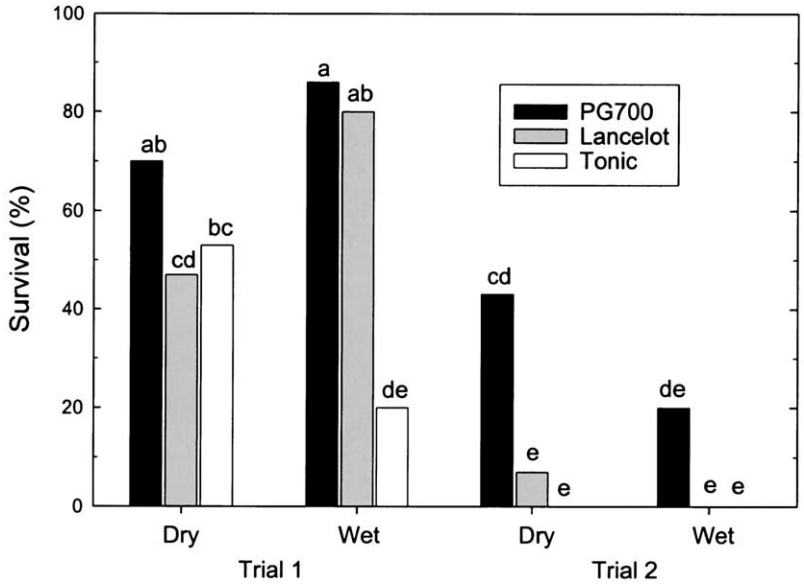

Fig. 1. Survival of plantain cultivars following exposure to −12°C for 3 h in controlled environment chambers. Bars superscripted by different letters are significantly different at $P < 0.05$ as determined by protected LSD.

reduction in shoot TNC-free dry weight (data not shown). There was a significant trial × water interaction for shoot TNC-free dry weight, with drought reducing shoot TNC-free dry matter in Trial 2 but not in Trial 1. Both shoot and root TNC content decreased by about 40% under drought (data not shown). There were no significant cultivar × water interactions.

PG700 had the least shoot biomass at the first and second harvests in both trials (Fig. 2) but essentially caught up with the two commercial cultivars during the cold-hardening period so that there was no significant difference among cultivars when the freezing treatment was applied. In contrast, PG700 root biomass was always less than the other two cultivars as was its total dry weight. In Trial 1, growth of TNC-free dry matter in shoots ceased during the cold-hardening period for Lancelot and Tonic, whereas, PG700 shoots continued to accumulate TNC-free biomass (Fig. 3). In Trial 2, however, all cultivars accumulated TNC-free dry matter in shoots during cold hardening and the relative rate of accumulation for PG700 was greater than in Trial 1 ($P < 0.01$). Relative growth rates for root TNC-free dry matter were highly variable and there was no difference among cultivars or between trials during cold hardening. At the beginning ($P = 0.04$) and end ($P < 0.01$) of cold hardening, the root:shoot ratio in Trial 1 was significantly greater than it was in Trial 2. Because of increased partitioning to shoots, root:shoot ratio decreased in Trial 2 from 0.61 at the beginning to 0.50 at the end of cold hardening. In contrast, during Trial 1, root:shoot ratio increased from 0.76 to 0.86 during the same period, indicative of a shift in partitioning from shoots to roots. At the end of cold hardening, Tonic had the greatest root:shoot ratio (0.79) followed by Lancelot (0.69) and PG700 (0.56) ($P = 0.02$).

Shoot nitrogen concentration decreased with each successive harvest ($P < 0.01$). Root N concentration also decreased significantly between the first and second harvests but remained relatively unchanged during cold hardening. Both root and shoot N concentration were significantly greater in Trial 2 than in Trial 1 at all harvests and for all cultivars (Table 1). In Trial 1, shoot and root N content increased between the first and second harvests, but there was no additional N taken up by the plants during cold hardening. The percentage of total plant N that was located in the roots increased from 30% at the beginning of cold hardening to 35% at the end. In Trial 2, both shoots and roots accumulated significant amounts on N during cold hardening, with shoot N content increasing 64% and root N content increasing 52%. In contrast with Trial 1, the percentage of total plant N in roots in Trial 2 decreased from 29% at the beginning to 27% at the end of cold hardening.

There was no significant trial × cultivar interaction for shoot N concentration. When averaged across trials, there was no difference among cultivars for shoot N concentration at the first harvest or following the drought treatment (Table 1). However, Lancelot had greater shoot N concentration than Tonic or PG700 at the end of cold hardening. In Trial 1, Lancelot had greater shoot N content than Tonic or PG700 at both the end of drought and the end of cold hardening. PG700 and Tonic did not differ from each other for N content at any harvest. In Trial 2, Tonic had greater shoot N content than PG700 at Harvest 1, with Lancelot being intermediate between the two. Lancelot shoots accumulated N more rapidly in Trial 2 than the other two cultivars so that following drought it was equal to Tonic and following cold hardening had greater shoot N content than the other two cultivars. PG700 had significantly lower N content than Tonic and Lancelot at both the second and third harvests (Table 1).

In both trials, PG700 had the greatest root N concentration at the initial harvest and at the end of cold hardening, while Lancelot and Tonic did not differ from each other. This was also true following drought in Trial

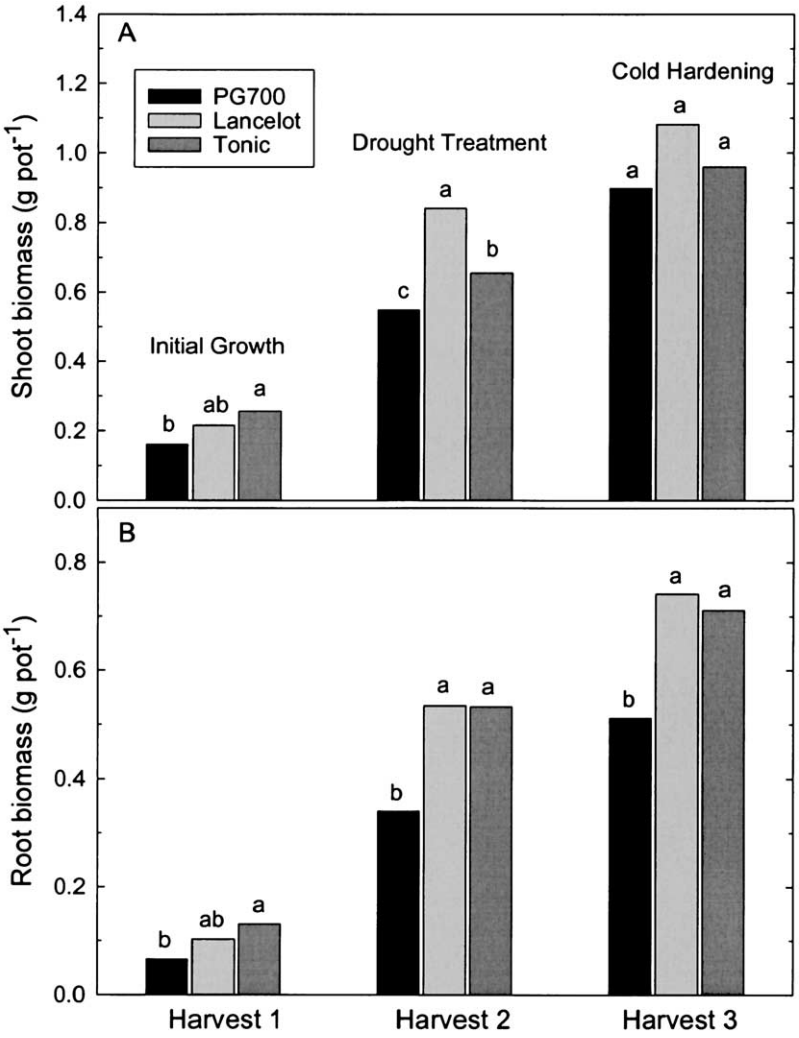

Fig. 2. Cultivar effects on shoot (A) and root (B) biomass following 3-wk growth in a controlled environment chamber (Initial Growth), after three 4- to 5-d drought cycles (Drought Treatment) and following a 3-wk cold hardening period (Cold Hardening). There was no significant cultivar × trial × water interaction, therefore, data are averaged across trials and drought treatments. Bars superscripted by different letters are significantly different at $P < 0.05$ as determined by protected LSD.

2. However, there were no differences among cultivars for root N concentration following drought in Trial 1. In Trial 1, there were no differences among cultivars for root N content at any harvest. However, in Trial 2, Tonic had greater root N content than PG700 and Lancelot at Harvest 1 and following cold hardening, but there were no differences among cultivars following the drought treatment.

Nonstructural Carbohydrates

Plantain appears to contain very little starch in either root or shoot tissues. Root starch averaged 2 mg g^{-1} dry weight or about 1% of TNC. Shoots contained about 19 mg g^{-1} starch or 10% of TNC. Shoot starch concentration decreased during cold hardening from 28 to 11 mg g^{-1}. Neither cultivar nor trial had any effect on shoot starch. Plant TNC concentration, therefore, was primarily a function of soluble sugar content. During cold hardening, shoot TNC concentration increased from 135 to 230 mg g^{-1} and root TNC concentration increased from 146 to 233 mg g^{-1}. Changes in TNC concentrations during cold hardening were similar in both trials. There was no significant difference among cultivars for shoot TNC concentration either before or after cold hardening. Tonic had greater root TNC concentration than Lancelot or PG700 which did not differ from each other (Fig. 4). Root TNC concentrations were almost identical at the end of cold hardening in each trial (231 vs. 235 mg g^{-1}, for Trial 1 and Trial 2, respectively). However, shoot TNC concentration was significantly greater in Trial 2 (215 mg g^{-1} in Trial 1 vs. 247 mg g^{-1} in Trial 2, $P < 0.01$).

DISCUSSION

The working hypothesis was that PG700 would exhibit reduced growth of shoot TNC-free dry matter and increased partitioning of nonstructural carbohydrates to roots during cold-hardening, resulting in increased freezing tolerance and survival. As expected, PG700 had greater freezing tolerance than the two commercial cultivars. However, the increased survival of PG700 was

Table 1. Nitrogen concentration and content of three plantain cultivars 3 wk following transplanting (Harvest 1), following drought treatment (Harvest 2) and following cold-hardening (Harvest 3). Data are averaged across drought treatments for Harvests 2 and 3. There was no drought × cultivar interaction for either N concentration or content.

	Concentration			Content		
	Harvest 1	Harvest 2	Harvest 3	Harvest 1	Harvest 2	Harvest 3
	mg g^{-1}			mg pot^{-1}		
			Shoot			
Trial 1						
PG700	34	18	13	8	12	13
Lancelot	34	20	19	10	20	20
Tonic	35	19	14	10	12	10
Trial 2						
PG700	48	25	21	5	11	17
Lancelot	50	23	27	7	16	30
Tonic	45	24	19	10	16	22
$LSD_{0.05}$	7†	3	3	4	5	5
			Root			
Trial 1						
PG700	28	11	13	2.7	4.9	8.8
Lancelot	21	11	8	3.1	7.1	7.4
Tonic	18	12	10	3.8	6.1	6.5
Trial 2						
PG700	43	18	24	1.3	5.6	7.3
Lancelot	36	15	15	1.4	5.4	7.8
Tonic	33	14	15	2.9	6.4	11.0
$LSD_{0.05}$	9	3	3	1.3	2.5	2.5

† LSD values are for comparisons among all root or shoot means within a given column.

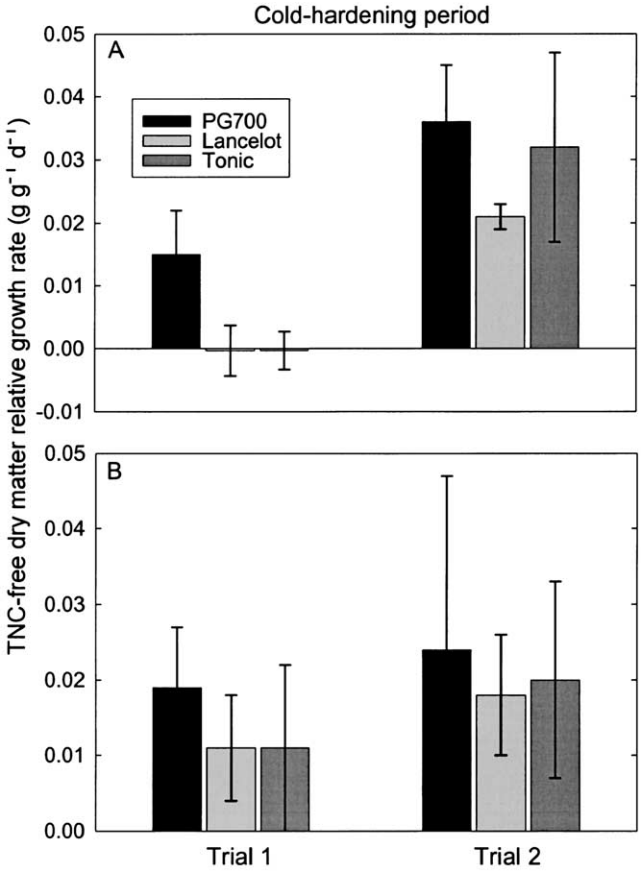

Fig. 3. Cultivar effects on shoot (A) and root (B) structural relative growth rates during the cold hardening period. TNC-free dry matter was determined by subtracting total nonstructural carbohydrates from total dry matter and differentiates structural growth from the accumulation of storage compounds. Bars superscripted by different letters are significantly different at $P < 0.10$ as determined by protected LSD.

not due to reduced shoot growth during cold hardening or to differences in root:shoot partitioning. In fact, PG700 had the greatest shoot relative growth rate during cold hardening, the smallest root system, and the lowest root:shoot ratio of any cultivar. Although Lancelot and Tonic differed in freezing tolerance, they did not differ in root or shoot growth during hardening or in TNC accumulation in roots or shoots. None of the parameters measured could adequately explain the differences in survival among cultivars.

As might be expected from an unreleased germplasm, PG700 plants produced less shoot and root biomass than the commercial cultivars before cold hardening. However, the accelerated shoot growth during the hardening process allowed PG700 to catch up with the other cultivars so that there was no difference in shoot biomass when the freezing treatment was initiated. Even though reduced growth during the fall has been closely related to winter survival in alfalfa (Schwab et al., 1996; Cunningham et al., 2001), Haagenson et al. (2003b) identified germplasm with less fall dormancy but greater winter hardiness than a commercially available cultivar. It appears that plantain freezing tolerance can also be enhanced without sacrificing shoot growth at low temperatures, which is one of the characteristics that makes the species a desirable component of pasture systems.

In a previous experiment, Skinner and Gustine (2002) found that imposition of drought stress before, but not during, cold-hardening resulted in increased freezing tolerance of Lancelot in a controlled environment and of Tonic in the field. Many studies have also shown that drought and low temperatures induce similar physiological and morphological adjustments including variations in enzyme activity, accumulation of soluble components, alterations in membrane composition, and induction of specific genes (Cattivelli et al., 1996). This is to be expected since cell dehydration is the primary cause of

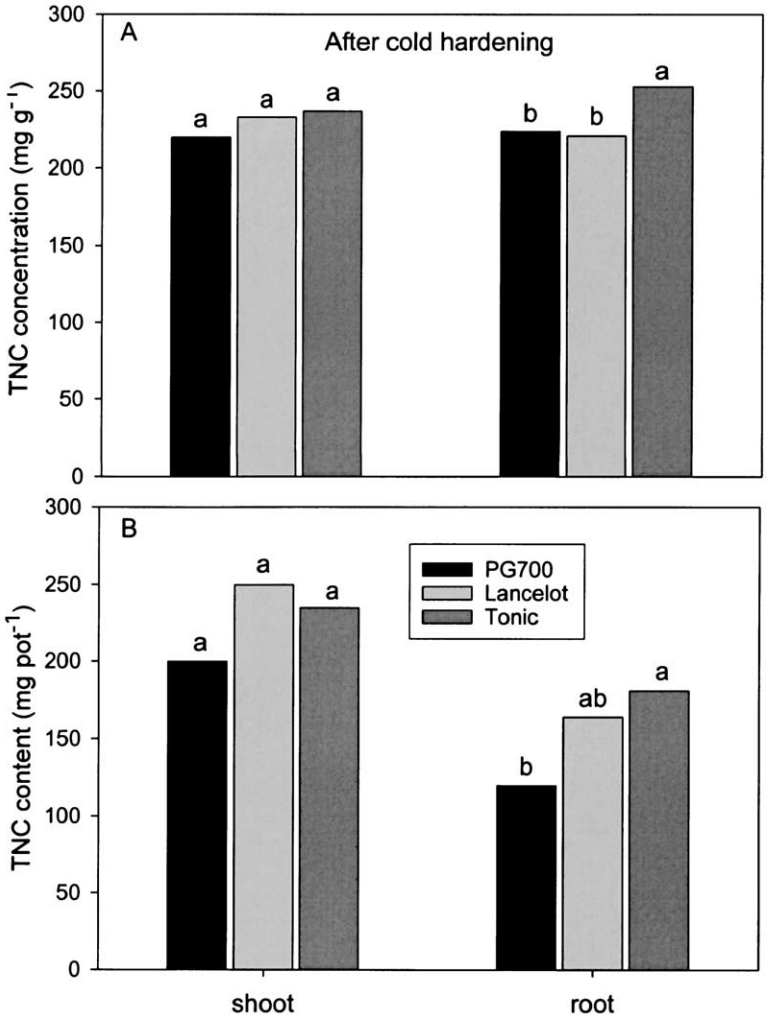

Fig. 4. Cultivar effects on tissue nonstructural carbohydrate concentration (A) and content (B) following a 3-wk cold hardening period. There was no cultivar × drought × trial interaction, therefore, data are averaged across drought treatments and trials. Bars superscripted by different letters are significantly different at $P < 0.05$ as determined by protected LSD.

freezing injury (Siminovitch and Cloutier, 1983). However, in this study, the effects of drought on freezing tolerance were mixed. In Trial 1, survival of Tonic was enhanced by the previous drought stress as expected, but survival of Lancelot was reduced. There was no obvious reason why the two varieties should have differed in their response to drought. In Trial 2, survival was numerically higher in the drought treatment for Lancelot and PG700, although survival was low enough and variability great enough that significant differences could not be detected.

The large difference in survival between trials was unexpected since growth chamber temperature, humidity and photoperiod, and experimental protocols were identical for the two trials. Nevertheless, overall survival was only 11% in Trial 2 compared with 59% in Trial 1. Although none of the expected physiological parameters were related to genetic differences in freezing tolerance, they did correspond well with differences in survival between trials. Plants in Trial 2 were initially smaller than in Trial 1, but overall growth and especially shoot growth was more rapid throughout the drought and cold-hardening treatments in Trial 2. Whereas, in Trial 1 only PG700 had a positive relative growth rate for shoot TNC-free dry matter during cold hardening, in Trial 2 all cultivars exhibited significant accumulation of shoot TNC-free biomass. The root:shoot ratio was greater in Trial 1 than in Trial 2 at all harvests and increased in Trial 1 from 0.76 at the beginning to 0.86 at the end of the cold hardening, indicating that biomass was preferentially partitioned to roots during that period. Even PG700, which exhibited increased shoot biomass during cold hardening, saw an increase in root:shoot ratio during the hardening period in Trial 1. In contrast, in Trial 2, root:shoot ratio for all cultivars decreased on average from 0.61 to 0.50 during cold hardening. In addition, in Trial 1, there was no net N uptake during cold hardening and there was some indication of net N translocation from shoots to roots. In Trial 2, both roots and shoots accumulated N during cold hardening, with the greatest accumulation rate occurring in shoots. Tissue N concentration was also greater in Trial 2 than in Trial 1 at all harvests and in both roots and shoots.

The combination of vigorous shoot growth at the expense of root growth during cold hardening and reduced survival in Trial 2 was consistent with the hypothesis that reduced fall dormancy should adversely affect winter survival. The high N concentration at all harvests and continued N accumulation during the hardening period in Trial 2 suggests that N availability was not identical between trials. Although N was applied weekly with irrigation water and was assumed to be adequate to support growth of the young seedlings, the quantity applied was not controlled. Pots were watered to saturation during the initial growth period and it is possible that more N was applied during Trial 2 than during Trial 1. For whatever reason, the N accumulation dynamics during the two trials were significantly different. Nitrogen fertilization is known to increase shoot relative to root growth (Davidson, 1969; Hilbert, 1990) and has also been shown to reduce freezing tolerance (Tyler et al., 1981).

The 7% reduction in photosynthetically active radiation in Trial 2 compared with Trial 1 might also have affected plant growth, partitioning, and freezing survival. Reduced light levels could be expected to reduced overall plant growth, increase partitioning to shoots relative to roots, and reduce TNC accumulation. In this experiment, root:shoot ratio was lower in Trial 2, consistent with the expected effects of both high N and low light. However, TNC concentration and whole plant relative growth rate were higher in Trial 2, which was not consistent with light limited growth. This suggests that the 7% reduction in photosynthetically active radiation was not the primary factor driving the growth differences that were observed between trials.

In this experiment, the reduced survival in Trial 2 appeared to be related to the reduced ability of the plants to acclimate to low temperatures by preferentially allocating resources to roots rather than shoots. It is interesting, however, that cultivar freezing tolerance was inversely related to root:shoot ratio in both trials. All cultivars preferentially accumulated biomass in roots during cold hardening in Trial 1 and preferentially accumulated biomass in shoots in Trial 2, but relative differences among cultivars in partitioning between roots and shoots did not appear to contribute to differences in freezing tolerance. In a study examining defoliation effects on winter survival of alfalfa cultivars possessing contrasting fall dormancy, Haagenson et al. (2003a) concluded that factors other than those that were positively associated with genetic differences in winter hardiness were responsible for regulating defoliation-induced changes in winter survival. Here I have obtained similar results, suggesting that factors associated with environmentally induced changes in winter survival are not necessarily related to genetically induced changes in freezing tolerance, nor are they necessarily mediated by the same physiological processes.

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